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TITLE: TAF1, From a General Transcription Factor to Modulator of Androgen Receptor in Prostate Cancer

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14. ABSTRACT The androgen receptor (AR) is a ligand-activated transcription factor that is essential for development and progression of prostate cancer. Using the N-terminus of AR as bait in the repressed transactivator yeast two-hybrid system, TATA binding protein-associated factor 1 (TAF1) was identified. TAF1, a multifunctional protein that contains acetylation, ubiquitin activating and kinase domains, can interact with several proteins to promote or suppress gene transcription. In the present study, using GST pull-down assay we confirmed that TAF1 binds to NTD of AR through the E1/E2 and HAT domains. We demonstrated by co-immunoprecipitation and ChIP assays that TAF1 and AR bind in the nucleus and associate with an androgen response element (ARE) at the proximal promoter of the prostate specific antigen (PSA) gene in the presence of androgen. In addition, we found that TAF1 acts as a coactivator and enhances AR transcriptional activity through its NTK and E1/E2 domains without influencing the general transcriptional activity of a non-androgen responsive promoter. By using in-cell and in-tube ubiquitination assays, our finding also suggested that TAF1 could ubiqinate AR.

15. SUBJECT TERMS
Androgen receptor, Prostate cancer, transcription

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INTRODUCTION

Prostate cancer is the most commonly diagnosed non-skin cancer in men and one of the leading causes of cancer death. Androgen withdrawal therapies are still the most effective treatment for advanced disease, although it eventually progresses to the lethal castration-resistant stage. Evidence suggests that most cells in castration-resistant tumors retain androgen receptor (AR) that maintains its transcriptional activity through alternative pathways (1-7). Using the N-terminal domain of AR (NTD) as bait in the repressed transactivator yeast two-hybrid system (RTA), TATA binding protein associated factor 1 **(**TAF1) was detected (8). TAF1 is a multifunctional protein that contains acetylation (HAT), ubiquitin activating/conjugating (E1/E2) and bipartite, kinase domains consisting of N- and C-terminal kinases (NTK and CTK, respectively). TAF1 is capable of autophosphorylation as well as specific phosphorylation of TFIIF (9), p53 (10), and Mdm2 proto-oncogene (11). TAF1 binds and modulates transcriptional activity of proteins, such as c- Jun (12), the Mdm2 (13), and cyclin D1 (14) that they can also influence AR activity and hence prostate cancer progression (15-17). The focus of this study is to identify if and how TAF1 modulates AR transcription, and to determine the role of TAF1 in prostate cancer progression. In particular, this research proposal will test the hypothesis that TAF1 directly modulates AR activity, and aids in the development and maintenance of castration-resistant prostate cancer. We are testing this hypothesis with the following four specific aims:

Specific Aim 1: To determine whether the TAF1/AR interaction specifically modulates AR transactivation

Specific Aim 2: To map functional interaction site(s) of AR and TAF1

Specific Aim 3: To determine the mechanisms through which TAF1 coactivates AR

Specific Aim 4: To assess the expression profile of TAF1 versus AR in prostate tumors:

a) To characterize TAF1 and AR expression at mRNA and protein levels in the *Pten*$^{-/-}$ prostate-specific knock out mouse model.

b) To determine the expression level of TAF1 and AR in malignant versus benign human prostate tissues.

PROGRESS AND RESULTS

A. Summary of last year report (specific aim 1)

We have demonstrated that TAF1 interacted with AR within nucleus of LNCaP cells, a prostate cancer cell line that expresses AR. Using ChIP assay, we also showed that TAF1 associated with AR at the prostate specific antigen (PSA) promoter in the presence of hormone. To assess if TAF1 modulates AR transcriptional activity, transactivation assays in prostate cancer cells were performed. TAF1 was either over-expressed (pHA-TAF1, kindly given by Dr. Tjian and Dr. Wong) or knocked down (siRNA duplexes; AAGACCCAAACAACCCCGCAT-3´and 5´-AACTACGACTACGCTCCACCA-3´) in prostate cancer cells and luciferase assays were performed. The results indicated that the expression level of TAF1 is directly correlated to AR activity only in the presence of hormone, when the receptor has been activated (Fig. I, appendix).

Since TAF1 is a member of the general transcription machinery complex it is expected that it influences on promoters of other genes. In our transactivation assays, we also found that the non-androgenic reporter (thymidine kinase-renilla (pRLtk-renilla)) is also modulated by TAF1 in a similar manner as androgen responsive reporters (Fig. IIB, appendix). To differentiate the effect of TAF1 on AR from its general effects on transcription and to determine which TAF1 domains are specifically involved in AR activation, we cloned various domains of TAF1 and repeated the luciferase assay in LNCaP cells. In contrast to full-length TAF1, TAF1 domains had no effect on the generic renilla construct with pRL-tk promoter, implying that general transcription is not affected. By comparison, while HAT and CTK domains had no significant effect on AR activity, NTK and the E1/E2 domains of TAF1 did enhance AR activity in a ligand dependent manner. NTK significantly enhanced AR transactivation by 2.4-fold, which is almost as much as the full-length TAF1. However, E1/E2 domain had even greater effect, enhancing AR activity over 3.4-fold (Fig. II, appendix).

In 2008, the specific aims 2 and 3 were tested and the results are discussed as follows:

B. Specific Aim 2: To map functional interaction site(s) of AR and TAF1

TAF1 interacts with the N-terminus of AR mainly through its HAT and E1/E2 domains

To confirm the AR/TAF1 interaction and to determine the domains involved, GST pull-down assays were performed using GST-fusion protein with AR/NTD_{1-559}, $DBD_{541-665}$, or $LBD_{649-919}$ (Fig. 1). Purified GST-AR domains were analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 1A). Equimolar amounts of non-degraded protein were determined for each of the GST protein products (*) and used in pull-down assays to assess relative TAF1 binding to AR domains, as described before (8). Radiolabeled TAF1 and TAF1 truncated domains were generated *in vitro* and verified by SDS-PAGE followed by autoradiography (Fig. 1B). The presence of multiple bands for radiolabeled proteins is due to several ATG sites within hTAF1 cDNA. Radiolabeled TAF1 (Fig. 1B, lane 1) was allowed to interact with GST protein or GST-AR fragments bound to agarose beads. As shown in Fig. 1C (top row), TAF1 did not interact with GST alone (lane 2), whereas it did bind to all domains of AR (lane 3-5) with the strongest interaction with the NTD of AR (lane 3).

GST pull-down experiments were repeated with radiolabeled TAF1 domains (NTK, HAT, E1/E2, and CTK proteins, Fig. 1B) to identify domains essential for interaction between TAF1 and AR (Fig. 1C, rows 2-4). All domains except the NTK of TAF1 bind to GST fusion AR proteins with different affinities. We performed the above pull-down assay with two more independent experiments and calculated an averaged percentage of total input bound to the GST–AR domains ([CNT∗ mm2] bound/[CNT∗ mm2] input), as described before (8). A summary of the quantified GST pull-down data (percentage of the input bound) for these TAF1 truncations and the full-length protein is shown in Fig. 1D. Similar to the full-length protein, HAT and E1/E2 domains interact most strongly with the NTD of AR, but the HAT domain binds this region with 1.5-times higher affinity. The CTK domain that was originally pulled out with the RTA assays with N-terminus of AR as a bait, also strongly interacts with DBD and LBD domains of AR. Taken together, these data demonstrate that TAF1 interacts directly with AR and mapping the interaction domains of TAF1 and AR suggests that the HAT, E1/E2 and CTK

domains of TAF1 are all involved in binding to AR. The pattern of binding of HAT and E1/E2 domains is similar to that seen with full length TAF1.

C. Specific Aim 3: To determine the mechanisms through which TAF1 coactivates AR

TAF1 and AR phosphorylation

To explore the molecular mechanism by which TAF1 enhances AR transcriptional activity, we tested the effect of apigenin, a TAF1 protein kinase inhibitor (10, 11), on AR transcriptional activity. Using MTS assay, we first found that apigenin at 5 μM did not influence LNCaP cell viability (data not shown). This concentration was used to treat LNCaP cells transfected with the PSA-Luc reporter and luciferase assays were performed. As shown in Fig. 2A, AR activity is suppressed more than 4-fold in the presence of apigenin. To show whether TAF1 can phosphprylate AR, *in vitro* kinase activity was performed. We were able to show that TAF1 autophosphorylates itself and the DNA binding domain of AR can be phosphorylated with ERK/MAPK, consistent with others (9, 18, 19) (Fig. 2B). However, we could not detect AR phosphorylation by TAF1 (data not shown).

Androgen receptor is ubiquitinated by TAF1

Since NTK does not bind to AR and the CTK and HAT domains do not enhance AR transcriptional activity, we focused on the E1/E2 domain, which binds to AR and has the most profound effect on its transactivation. Ubiquitination is a posttranslational modification that mediates the covalent conjugation of ubiquitin to protein substrates. The functional role of ubiquitination was originally considered to be targeting proteins to the proteasome for degradation. However, it is now known that ubiquitination regulates many other processes in the cell, including membrane trafficking, DNA repair, and transcription (20). AR is also a direct target for mono and poly-ubiquitination (16, 21). To address whether TAF1 can ubiquitinate AR, LNCaP cells were co-transfected with pHis_6-Ubiquitin and either pHA-TAF1 or empty vector. Cells were then treated with 10% FBS RPMI followed by 6h treatment with vehicle or MG132, a proteasome inhibitor. To show the His-ubiquitin conjugated status of AR in the presence and absence of MG132, after saving 5% input (Fig. 3A), His-conjugated proteins were purified followed by a Western blot with AR and His antibodies. Fig. 3B shows that in the absence of MG132, there is no His-conjugated AR when TAF1 is not overexpressed (lane 1 & 5). Whereas, ubiquitinated AR is seen once TAF1 is overexpressed. (lane 2, arrow). As expected, in the presence of proteasome inhibitor, the total amount of conjugated AR is increased irrespective of TAF1 status level (lane 3, 4, 7 & 8). However, there is a drastic increase in the level of ubiquitinated AR once TAF1 is overexpressed (lane 4 & 8 versus 3 & 7). The results indicate that TAF1 facilitates AR ubiquitination in a spectrum from mono to poly-ubiquitin form.

TAF1 ubiquitinates AR *in vitro*

To confirm the ubiquitination of AR by TAF1 and to assess whether TAF1 is able to directly ubiquitinate AR, an in-tube ubiquitination assay was performed (22). As an abundant source of TAF1, HeLa cells were subjected to pull-down with anti-TAF1 antibodies and immobilized on beads. Protein extracts from LNCaP cells were incubated with radiolabeled ubiquitin, 10 nM ATP and TAF1 immobilized on agarose beads or beads alone at room temperature. After an hour, AR was immunoprecipitated and subjected to Western blot analysis. Fig. 3B shows that

AR can be ubiquitinated *in vitro* (out of cells) (lane 2) and that the enriched amount TAF1 enhances the amount of both mono and poly ubiquitinated forms AR (lane 3).

KEY RESEARCH ACCOMPLISHMENTS

1. TAF1 interacts with AR within prostate cancer cells and associates on the promoter of the PSA gene.
2. TAF1 binds to the N-terminal domain of AR. This interaction is mainly through HAT and E1/E2 domains of TAF1
3. The NTK and E1/E2 domains of TAF1 specifically enhance AR transcriptional activity and that mechanism is different from the role of TAF1 on general transcription machinery.
4. TAF1 can ubiqutinate AR inside a prostate cancer cell line and in test tubes.

REPORTABLE OUTCOME

1. A summary of the above data has been presented in the following meetings:
2. Poster presentation: The Endocrine Society's meeting 89th Annual Meeting, Toronto, Canada, June 2007
3. Oral presentation: The 6th Annual Canadian Prostate Cancer-BioNet Meeting, Montreal, Canada, April 22-24, 2007
4. Poster presentation: The Endocrine Society's meeting 90th Annual Meeting, San Francisco, CA, June 2008
5. Oral presentation: The urologic science meeting, Vancouver, Canada, June 12, 2008
6. Poster presentation: the 4th PacRim Breast and Prostate Cancer, Whistler, Canada, August 2008
7. Manuscript in preperation for the Molecular Endocrinology journal:
   Tavassoli P., Wafa L. A., Cheng H., Zoubeidi A., Fazli L., Gleave M., Rennie P. S. TATA binding protein-associated factor 1 (TAF1) can bind and differentially enhance androgen receptor transcriptional activity via its ubiquitin activating and conjugating domain

CONCLUSION

The molecular mechanisms responsible for the development of CR prostate cancer are largely unknown, but typically they do not appear to involve the loss of AR expression (23). The up-regulation of AR-target genes and over-expression of AR at the protein and mRNA levels support the notion that AR activity is altered in castration-resistant states (24-27). There are a variety of molecular alterations that could lead to continued or amplified AR signaling following surgical or medical castration. Recent evidence suggests that AR-specific gene regulation may

occur through interactions with unique coregulatory proteins. Since the N-terminus of AR (AR-NTD) is the least conserved, protein interactions in this region may dictate receptor-specific coregulation capacity. Using the RTA yeast two-hybrid system TAF1 was identified as a novel AR-NTD-interacting protein (8) and this interaction was confirmed with the full-length TAF1 using GST pull-down assays (Fig. 1C). Mapping of the TAF1 and AR interacting domains showed that HAT and E1/E2 domains bind strongly to AR.NTD mimicking the full length TAF1. The CTK domain that was originally isolated by the RTA system interacts with all AR domains but most strongly with the AR.DBD. In contrast, NTK does not have affinity for any AR domains, further indicating the specificity of these interactions (Fig. 1C & D). It has been reported by others that the N-terminus of TAF1 binds to the concave surface of TATA binding protein (TBP) and consequently inhibits TBP/ TATA box contact, thus repressing transcription (12, 28). However, binding of activators, such as c-Jun with the N-terminus of TAF1 releases this inhibition resulting in transcription initiation (29). Accordingly, the ability of TAF1 to interact with AR through multiple domains other than NTK suggests that TAF1 may play a role in modulating AR and one can speculate that upon interaction with AR, NTK release from the concave surface of TBP will initiate transcription. This hypothesis is supported by the fact that upon overexpression of TAF1 in both PC3 and LNCaP cells in the presence of nuclear AR (hormone induced activation), AR activity is increased (Fig. IA&B in appendix), whereas siRNA knock down of TAF1 suppresses AR activity (Fig. IC&D in appendix).

Co-immunoprecipitation assays of the nuclear extracts of LNCaP cells demonstrate that the interaction between AR and TAF1 is enhanced in the presence of hormone (R1881) (data was shown in last year report). Since TAF1 is a component of the general transcription machinery within the TFIID complex and directly associates with AR to modulate AR activity, we explored whether TAF1 co-localizes with AR at the PSA promoter. Using ChIP assays with LNCaP cells, we found that TAF1 is associated with an ARE in the proximal promoter of the PSA gene (data was shown in last year report). About 18% of genes of a hamster cell line are TAF1-dependent genes (30, 31). The essential nature of TAF1 can be attributed to its broad requirement during RNA-pol-II-dependent transcription (32). In our studies, we also found that TAF1 can modulate the transcription of non-androgen responsive reporters (Fig. IIB, appendix). To show that there is a specific effect of TAF1 on AR, various domains of TAF1 were tested for their effect on androgen and non-androgen responsive reporters in transient transfection assays (Fig. II, appendix). Although TAF1 is a component of the transcriptional machinery, it appears to be a specific coactivator of AR, enhancing AR transcription through its NTK and E1/E2 domains (Fig. IIA, appendix). In contrast, unlike the full-length TAF1, these truncated proteins did not modify generic promoters such as ptk-Renilla (Fig. IIB, appendix).

The AR is a substrate for phosphorylation, acetylation and ubiquitination (16, 21, 33-35). This makes AR as a potential substrate for TAF1 that possesses all the above enzymatic activities. We did not pursue to determine whether TAF1 can acetylate AR because no significant changes on AR activity were seen in transfection assays when the HAT domain was overexpressed (Fig. IIA). However, we have demonstrated that apigenin, a TAF1 kinase inhibitor (10, 11) can suppress AR transcriptional activity by 4-fold (Fig. 2A). To further explore the TAF1 kinase activity on AR, *in vitro* kinase assays were performed. Although we could show that TAF1 can autophosphorylates itself (Fig. 2B), but we were unable to show AR phosphorylation by TAF1. This could be due to inadequacy of cell-free assays, which are not representative of cell biology.

It has been reported that TAF1 can mono-ubiquitinate histone 1(H1) and poly-ubiquitinate p53 (10, 32). Since E1.E2 has the most profound effect on AR activity, we also sought to determine whether ubiquitination of AR can be increased as a consequence of TAF1 overexpression. Interestingly, in the presence of proteasome inhibitor (MG132) and overexpression of ubiquitin protein, full length-TAF1 enhances the total amount of ubiquitinated AR (Fig. 3A, lane 4 & 8). This was further validated using an *in vitro* ubiquitination assay (Fig. 3B, lane 3).

Taken together, we have demonstrated that TAF1 binds to the N-terminus of AR mainly through its HAT and E1/E2 domains and associates with AR in the nucleus and at the PSA promoter. In addition, we showed that TAF1 through its NTK and E1/E2 domains can affect AR transactivation without influencing the general transcription machinery. Finally, we have demonstrated that TAF1 is able to ubiquitinate AR both *in vivo* and *in vitro*. Currently, we are assessing the level of TAF1 expression in human prostate cancer samples using our gleason grade and neo-adjuvant tissue microarray (aim 4).

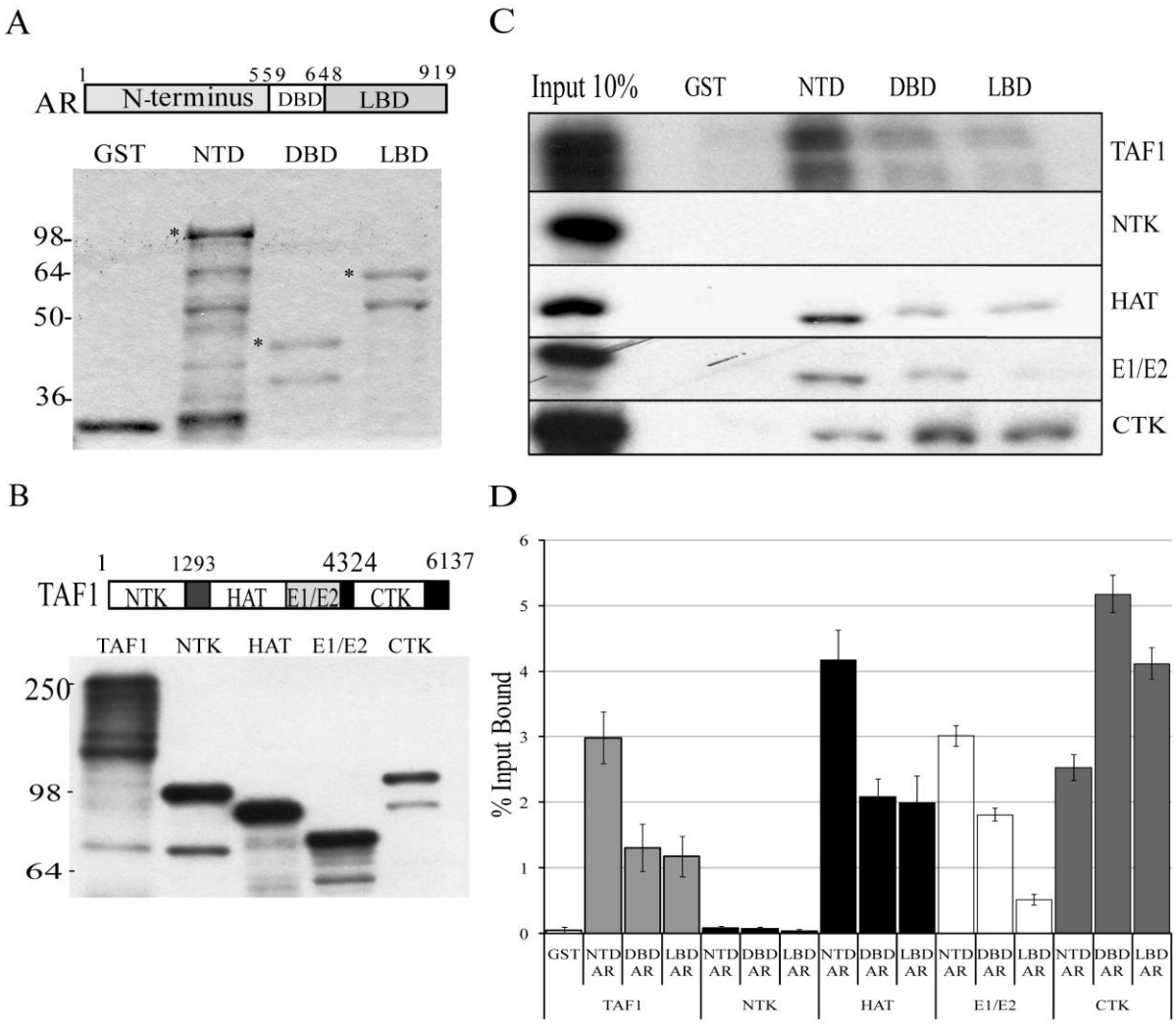

Figure 1. TAF1 binds AR through HAT and E1/E2 domains *in vitro*.
A. GST-fused AR domains (N-terminus =NTD, DNA binding domain =DBD, ligand binding domain =LBD) were expressed in E. Coli BL21 and purified using glutathione beads. Fusion protein-bound bead volumes were titrated, eluted with sample buffer and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. The eluent in each case was run alongside known amounts of BSA (ranging from 250 to 1000 ng) to generate a standard curve for protein concentrations. Equimolar amounts of non-degraded proteins (*) were used in GST pull-down assays. **B.** [^{35}S] Radiolabeled TAF1 and its domains (N-terminal kinase (NTK), Histone acetylation (HAT), Ubiquitin activating conjugating (E1/E2), and C-terminal kinase (CTK) were generated using *in vitro* Transcription/Translation kit. **C.** GST pull-down assay. Equivolume of [^{35}S] labeled TAF1, [^{35}S] labeled NTK, [^{35}S] labeled HAT, [^{35}S] labeled E1/E2, [^{35}S] labeled CTK were incubated with GST-AR fragments bound to agarose beads. GST alone coupled to agarose beads was used as negative control. **D.** Dried gels were also analyzed using a Phosphorimaging screen. Quantity One software was used to obtain data (counts/mm2) for radiolabelled protein bands. All pull-downs were done in triplicate, averaged, and normalized as a function of the percentage input bound.

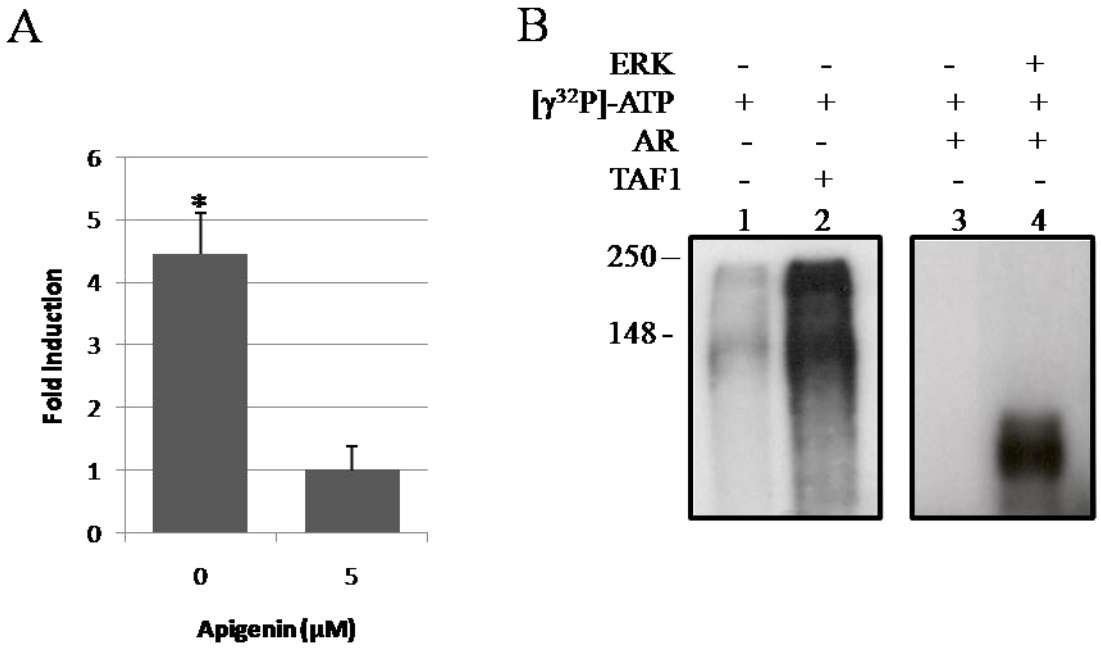

Fig. 2: A) LNCaP cells were co-transfected with 0.7 µg/well pPSA-Luc and 0.1 µg/well pRLtk-renilla. Transfected cells were grown in the presence of 1 nM R1881 and either 5 µM apigenin or vehicle for 24 h before harvesting for luciferase assay. Luciferase units were normalized to protein levels and fold–induction was calculated. All values are given as the mean (± SEM) of triplicate readings. Graphs are representative of the 3 independent experiments. * indicates a $p<0.05$ compared to empty vector control. B) HeLa cells were subjected to pulldown with normal IgG (lane 1) or anti-TAF1 (lane 2) antibodies immobilized on beads and incubated in kinase buffer (1M Hepes pH 7, 1M $MgCl_2$, 1M $MnCl_2$, 100 mM Na_3VO_4, 10X microcytin) with the presence of [γ^{32}P]-ATP at 30ºC for 20 min. Mock enzyme (lane 3) or ERK (lane 4) were incubated in kinase buffer with the presence of [γ^{32}P]-ATP and GST fusion of DBD of AR at 30ºC for 20 min.

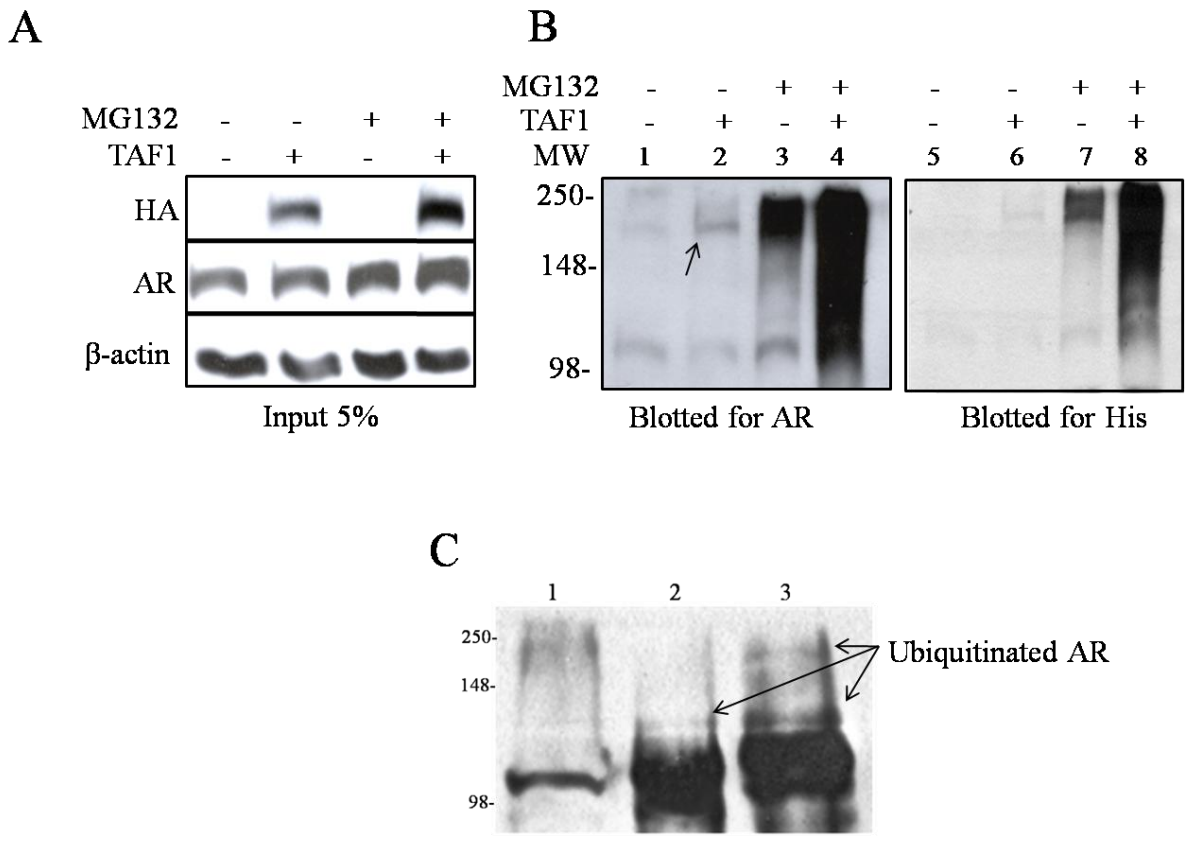

Figure 3. TAF1 ubiquitinates AR. LNCaP cells were transfected with 2 µg pHis$_6$-ubiquitin and either 6 µg of pHA-TAF1 or empty vector. Cells were then treated with 5% FBS RPMI followed by ±10 µM MG132 for 6h. After harvesting and lysing the cells in RIPA buffer, 5% of cell lysate was used as an input (A) and the remainder was mixed with 50 µl Ni^{2+}-NTA-agarose beads. The mixture was rotated at 4ºC for 3 h and then affinity pulled down followed by Western blot analysis for AR and His (B). The input was blotted for HA, AR and β-actin. C) TAF1 was imunopreciptated from nuclear extracts of Hela cells. LNCaP cell lysate was incubated with 1 mM ATP, ubiquitin and either TAF1 immobolized on agarose beads (lane 3) or beads alone (lane 2) in 20 µl 0.4 M HEMG. After 1 h incubation at room temperature, AR was immunoprecipitated followed by a Western blot for AR. Lane 1 = Input (5%).

APPENDIX

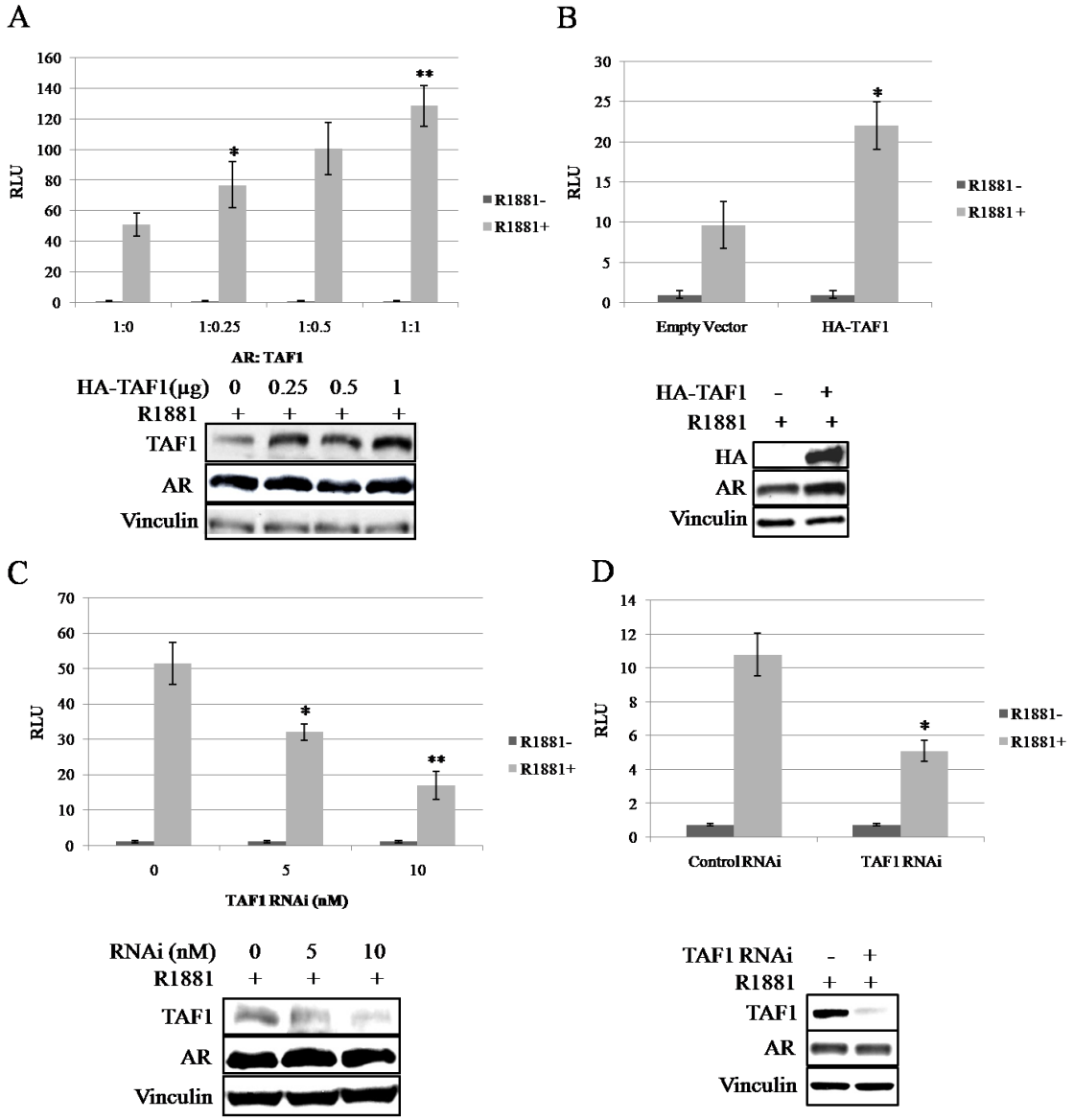

Figure I. TAF1 modulates AR transactivation.

PC3 cells were co-transfected with 1.5 µg/well full length AR (pAR_6) and 0.2 µg/well pARR3-tk-Luc reporter, 0.1 µg/well pRLtk-renilla, and increasing amounts of pHA-TAF1 **(A)** or increasing amounts of TAF1 RNAi duplexes **(C)**. LNCaP cells were co-transfected with 1 µg/well pPSA-Luc, 0.1 µg/well pRLtk-renilla and 1 µg/well pCS2+HA-hTAF1 **(B)** or 10 nM TAF1 RNAi duplexes **(D)**. Transfected cells were growing in the presence or absence of 1 nM R1881 for 24 h (A, B) or 48 h (C & D) before harvesting for luciferase assay and Western blot analysis. Luciferase units (RLU) are expressed relative to protein values for each sample. All luciferase values are given as the mean (± SEM) of triplicate readings. Graphs are representative of the 3 independent experiments. * indicates a $p<0.05$ compared to empty vector control. ** indicates a $p<0.05$ compared to *.

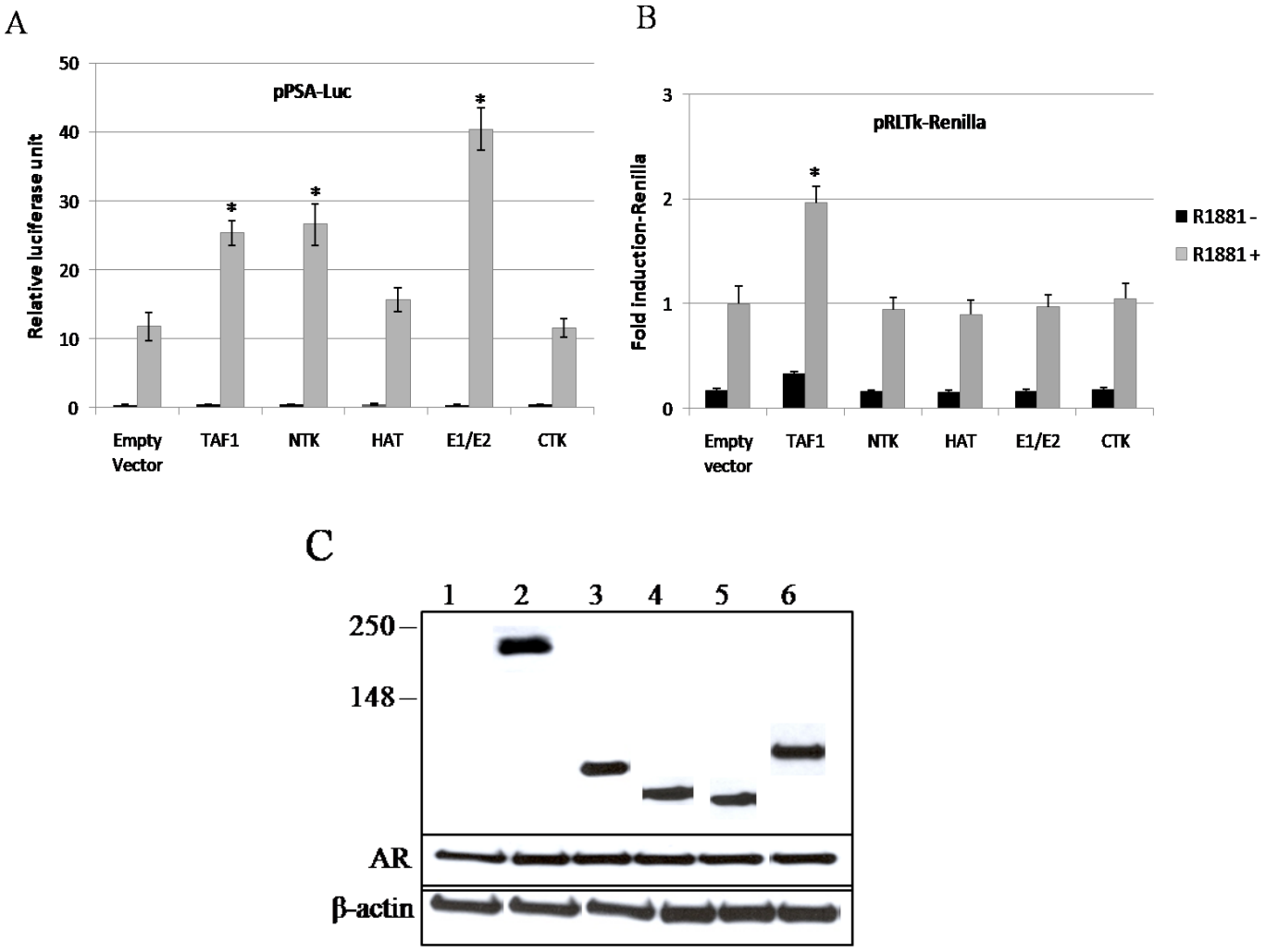

Figure II. N-terminal kinase and ubiquitin activating/conjugating domains of TAF differentially enhance AR transactivation. LNCaP cells were co-transfected with either pHA-TAF1 or one of its four domains (pV5-NTK, pV5-HAT, V5-E1/E2, or pV5-CTK) (1 ug/well) and the pPSA-Luc and pRLtk-renilla. Transfected cells were growing in the presence or absence of 1 nM R1881 for 24 h before harvesting for luciferase assay. A) luciferase units were normalized to protein. B) fold-induction of renilla units were plotted against empty vevtor, TAF1 or its domains. * indicates a $p<0.05$ compared to empty vector control. C) Western blot analysis for AR, β-actin, empty vector (EV) (1), TAF1 (2), NTK (3), HAT (4), E1/E2 (5) and CTK (6).

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